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New Perspectives on Old Questions

Engineered microenvironments to direct epidermal stem cell behaviour at single cell resolution

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Summary

Mammalian epidermis is maintained through proliferation of stem cells and differentiation of their progeny. The balance between self-renewal and differentiation is controlled by a variety of interacting intrinsic and extrinsic factors. Although the nature of these interactions is complex, they can be modeled in a reductionist fashion by capturing single epidermal stem cells on micropatterned substrates and exposing them to individual stimuli, alone or in combination, over defined time-points. These studies have shown that different extrinsic stimuli trigger a common outcome – initiation of terminal differentiation – by activating different signalling pathways and eliciting different transcriptional responses.

Introduction

The epidermis forms the outer covering of mammalian skin and is the subject of extensive research because of its physiological importance and ready accessibility (Hsu et al., 2014; Watt, 2014). It comprises a multilayered epithelium, known as the interfollicular epidermis (IFE), and associated hair follicles, sweat glands and sebaceous glands. Studies in mice have revealed the existence of multiple stem cell populations that differ in their location and the markers that they express. Under steady state conditions the progeny of these cells primarily repopulate the differentiation compartment in their immediate surroundings, such that a stem cell in the hair follicle will produce cells of the differentiated hair follicle lineages, not the

IFE, and vice versa. However, when the epidermis is damaged, reconstituted from single cells or genetically modified, stem cells exhibit a broader ability to differentiate into all the differentiated epidermal cell types (Schepeler et al., 2014; Blanpain and Fuchs, 2014). Epidermal stem cells thus exhibit plasticity in response to environmental cues (Donati and Watt, 2015).

There is considerable interest in the intrinsic and extrinsic signals that regulate epidermal stem cell fate and functionality (Figure 1). Many of the key epidermal transcription factors (Botchkarev, 2015) and their enhancers (Adam et al., 2015) have been identified. Micro RNAs and lncRNAs involved in the regulation of cell fate (Kretz et al., 2013; Lopez-Pajares et al., 2015) have been found and our understanding of the interplay between gene expression and chromatin organisation is increasing steadily (Mulder et al., 2012; Bao et al., 2015). In terms of potential extrinsic signals, these reflect the complex spatial organisation of the epidermis, the basement membrane on which it rests, and the multiple non-epithelial cell types present within the underlying connective tissue, known as the dermis (Figure 1). An additional consideration is that some cells are permanent residents, such as fibroblasts and cells of the peripheral nervous system, while others are transient visitors, such as cells from the blood. At this point, we have a comprehensive catalogue of intrinsic and extrinsic factors, and the challenge is to understand the significance of their interactions.

Although considering the environment might appear daunting, it is possible to take a reductionist approach, focusing on the interfollicular epidermis (IFE) (Figure 1). The IFE is essentially organised like a brick wall, with the stem cells in the bottom row and the differentiated cells in the upper rows. Stem cells in the basal layer are attached to an underlying basement membrane. Cells that move out of the basal layer undergo terminal differentiation, such that the further a cell has moved from the basal layer the further it has progressed along the programme of terminal differentiation.

A further advantage of studying the IFE is that it is possible to re-create the basic multilayered structure in culture. When human epidermal cells (keratinocytes) are seeded at clonal density on a feeder layer of 3T3 cells (Rheinwald and Green, 1975), they expand to form confluent sheets that retain a stem cell compartment. These sheets have been used for many years as an autologous treatment for burn injuries (O'Connor et al., 1981). The long-term persistence of the grafted sheets establishes that stem cells that have been expanded in culture retain in vivo functionality for many years (Green, 2008). Cultured keratinocytes thus provide an important experimental model for analysing stem cell niche interactions using stem cells from adult human tissue.

Dual role of ECM adhesion in regulating epidermal differentiation and tissue assembly

Soon after the technique for expanding human keratinocytes in culture was developed, it was observed that when the cells were disaggregated and suspended in semi-solid medium they withdrew from the cell cycle and underwent terminal differentiation (Green, 1977). Suspension-induced differentiation can be partially inhibited by adding extracellular matrix (ECM) proteins or anti-integrin antibodies to cells in suspension, establishing a role for cell-ECM interactions in regulating terminal differentiation (Adams and Watt, 1989). In addition, the onset of terminal differentiation in the basal layer of the epidermis is linked to upward migration because cells lose ECM adhesiveness, via downregulation of integrin expression, as part of the differentiation programme (Watt and Green, 1982; Adams and Watt, 1989).

These early studies established the dual role for ECM adhesion in controlling differentiation and tissue assembly. In addition, by using clonal growth of human keratinocytes as a quantitative measure of stem cells, it was possible to show that stem cells adhere more rapidly to ECM proteins and express higher levels of β1 integrins than basal cells with lower self-renewal ability (Watt, 2002). Downstream of β1 integrins the extracellular-signal-related kinase (ERK)/mitogen-activated

protein kinase (MAPK) pathway was found to be important for maintenance of the stem cell compartment (Watt, 2002).

Single epidermal stem cells on micro-patterned surfaces

One approach that can be taken to analysing the mechanism by which ECM adhesion controls the onset of keratinocyte terminal differentiation is to use micro-patterned substrates. These substrates are generated using photolithography or other techniques to stamp adhesive 'islands' that allow individual cells to adhere to a substrate; by changing island area and geometry it is then possible to control cell spreading and shape (Théry, 2010).

One of the earliest studies involving micro-patterned islands investigated the link between adhesion and anchorage dependent growth in fibroblasts (O'Neill et al., 1979). Using the same platform we showed that restricted spreading is a potent terminal differentiation stimulus for human keratinocytes (Watt et al., 1988). In more recent experiments we have exploited the protein resistance of poly(oligo(ethylene glycol methacrylate)) (POEGMA) brushes to achieve high fidelity patterning of single cells by soft lithography (Gautrot et al., 2010). ECM proteins are adsorbed onto the unprotected areas comprising gold-coated glass.

The power of controlling interactions between stem cells and their environment at single cell resolution in order to explore the downstream signalling events that lead to changes in cell fate decisions is now well established (Peerani and Zandstra, 2010). Several ground-breaking studies paved the way for that appreciation. These include the demonstration that when single human mesenchymal stem cells (MSC) are able to spread on ECM-coated micropatterned substrates they differentiate into osteoblasts, but when they remain rounded they differentiate into adipocytes, the switch in lineage commitment being controlled by endogenous RhoA activity (McBeath et al., 2004).

More recently we have used micro-patterned islands to map the downstream events that are responsible for the onset of terminal differentiation when human

keratinocytes are plated on islands that prevent cell spreading and thus identify the transcriptional response to biophysical cues. We confirmed that we could selectively enrich for stem cells on the basis of rapid adhesion to ECM-coated substrates (Connelly et al., 2010). On small (20 μm diameter) circular islands, keratinocytes remained rounded, and differentiated at higher frequency than cells that could spread on large (50 μm diameter) islands, thereby confirming the original findings of Watt et al. (1988). We found that the actin cytoskeleton mediated shape-induced differentiation by regulating serum response factor (SRF) transcriptional activity (Connelly et al., 2010). Knockdown of SRF or its co-factor MAL (Megakaryocytic Acute Leukaemia; also known as MRTF-A or MLK1) inhibited differentiation, whereas overexpression of MAL stimulated SRF activity and expression of markers of terminal differentiation such as involucrin and transglutaminase1. SRF target genes FOS and JUNB were also required for differentiation. We found that for differentiation two conditions were required: the presence of soluble serum-derived growth factors and restricted cell spreading. c-Fos mediated serum responsiveness, whereas JunB was regulated by actin and MAL.

Using the same platform we went on to show that inhibition of p38 MAPK activity also impaired SRF transcriptional activity and initiation of terminal differentiation (Connelly et al., 2011). Although p38 inhibition reduced histone H3 acetylation at the FOS and JUNB promoters, we found that the histone de-acetylase inhibitor trichostatin A (TSA) inhibited differentiation independently of SRF, leading us to speculate that there may be a role for global histone de-acetylation in repressing stem cell maintenance genes (Connelly et al., 2011).

We found that when human epidermal stem cells attach to micro-patterned substrates, the decision to differentiate does not depend on ECM concentration or composition (comparing fibronectin, laminin and collagen), nor on integrin clustering in focal adhesions (Connelly et al., 2010). It does, however, depend on the shape of the adhesive island, such that for a given area, the proportion of cells that differentiate is higher on a circular substrate than one that allows the cells to elongate (Connelly et al., 2010). In addition, it is not necessary to provide an ECM

coating over the entire surface area of each island. Keratinocytes do not differentiate on 40 μm diameter islands even when the centre is non-adhesive and the total ECM area only corresponds to a 20 μm diameter island (Tan et al., 2013b). Furthermore, when keratinocytes are captured on circular islands the surface hydrophilicity and charge of the surface do not influence differentiation; however, those physical properties do affect differentiation of keratinocytes on asymmetric, arc-shaped patterns (Tan et al., 2013b). The mechanism by which cells differentially respond to islands that have the same area but different shapes remains to be explored.

Epidermal stem cell responses on hydrogels

An important adjunct to studies of cells on micro-patterned substrates is to study cell responses to substrates that differ in bulk stiffness (Murphy et al., 2014). In a landmark study, human MSC were directed to differentiate by seeding on collagen-coated acrylamide hydrogels that differ in bulk stiffness and correspond to the true stiffness that cells sense in different tissues in the body (Engler et al., 2006). The physical properties of the hydrogel determined the differentiation pathway that was selected. Cells selected a neurogenic lineage on substrates with the lowest elasticity, an osteogenic lineage on the stiffest substrate, and a myogenic lineage on intermediate substrates. Substrate elasticity-directed differentiation is dependent on nonmuscle myosin II.

Given that MSC sense bulk stiffness to make cell fate decisions (Engler et al., 2006) and that the bulk stiffness of human skin is several orders of magnitude less than the glass substrates used to create micro-patterned islands, we investigated how human epidermal keratinocytes respond to ECM coated onto hydrogels of differing stiffness. We found that keratinocytes seeded on collagen or fibronectin-coated polyacrylamide of low elastic modulus (0.5 kPa) could not form stable focal adhesions or spread and underwent terminal differentiation within 24h (Trappmann et al., 2012). However, even though the cells remained rounded and differentiated, as in the case of small micro-patterned islands, the downstream signalling events

were different. On soft hydrogels keratinocytes differentiated as a result of decreased activation of the ERK MAPK signalling pathway, which in turn reflected the failure of β1 integrins to cluster in focal adhesions, and SRF transcription was not activated. Dextran penetration measurements indicated that polyacrylamide substrates of low elastic modulus were more porous than stiff substrates, which led us to hypothesise that the collagen anchoring points were further apart. Support for this conclusion came from the ability to control differentiation on collagen coated hydrogel-nanoparticle substrates by varying the distance between particles (Trappmann et al., 2012). We concluded that stem cells exert a mechanical force on collagen fibres and gauge the feedback to make cell-fate decisions.

In subsequent studies Wen et al. (2014) explored the concept that in addition to substrate stiffness, the tethering of ECM proteins and hydrogel porosity regulates stem cell differentiation. They found that varying substrate porosity did not significantly change protein tethering or the ability of human adipose-derived stromal cells and marrow-derived MSC to differentiate along adipogenic versus osteogenic lineages. Varying ECM tethering also had no effect on MSC differentiation. The authors therefore concluded that substrate stiffness regulates stem cell differentiation independently of protein tethering and porosity.

A further approach to understanding how keratinocyte-ECM interactions regulate terminal differentiation is based on the appreciation that although cell size is of the order of 10 μm diameter, the distance between focal adhesions in spread cells is in the nanometer range (Moore et al., 2010). Gautrot et al (2014) hypothesised that by altering the nanoscale properties of ECM coating, glass substrates might mimic the effect of soft hydrogels on keratinocyte spreading and differentiation. To test this, they created circular fibronectin-coated gold patterns with sizes ranging from 100 nm to 3 μm, surrounded by protein-resistant polymer brushes. Cells spread more on 3 μm patterns than on the smaller spacings and the proportion of involucrin-positive cells was lower. Differentiation on 100 nm nanopatterns was blocked by treatment with JNK and AP-1 inhibitors, implying that, as on soft acrylamide hydrogels, integrin clustering, the MAPK pathway, and associated JNK and AP-1 activation, control

keratinocyte terminal differentiation. The size of vinculin containing focal adhesions correlated with the size of the underlying patterns. On the small spacings vinculin was destablised and unable to mediate tension in developing focal adhesions. The authors concluded that impaired mechanotransduction, rather than impaired recruitment of proteins involved in focal adhesion formation, triggered differentiation.

Regardless of what cells 'sense' when plated on soft hydrogels and hydrogel-nanoparticle substrates, these studies do establish that different stimuli trigger differentiation via different signalling pathways, and that the same signalling pathway can be triggered by different extrinsic cues.

Regulation of differentiation by intercellular adhesive interactions

Although ECM adhesion clearly plays a central role in directing epidermal stem cell fate decisions, keratinocytes are virtually never found as single cells in vivo. Instead, epidermal integrity depends on intercellular adhesion mediated by several classes of adhesive junction (Simpson et al., 2011; Sumigray and Lechler, 2015). One of these is the adherens junction containing classical cadherin transmembrane receptors. P-cadherin and E-cadherin are expressed in the basal cell layer of the IFE, while in the suprabasal layers there is upregulation of E-cadherin and downregulation of P-cadherin. Desmosomal junctions are also essential for epidermal organisation and function and their transmembrane receptors are nonclassical cadherins known as desmogleins and desmocollins. As keratinocytes undergo terminal differentiation there are changes in the number and molecular composition of the desmosomal junctions, both at the level of transmembrane receptors and at the level of the desmosomal cytoplasmic plaque proteins (Johnson et al., 2014). In addition, cells in the epidermis form gap junctions, by which small molecules are transferred between cells (Blaydon and Kelsell, 2014), and, as they differentiate, tight junctions, which are important for the barrier function of the outermost layers (Sumigray and Lechler, 2015).

Assembly of adherens junctions and desmosomes is prevented by reducing the level of calcium ions in the cell culture medium yet, perhaps unsurprisingly, this has no effect on the terminal differentiation of single keratinocytes on micro-patterned islands (Connelly et al., 2010). Conversely, the adhesion-independent effect of overexpressing the E-cadherin cytoplasmic domain in promoting keratinocyte differentiation in suspension is through sequestration of β-catenin rather than a direct effect on cell-cell adhesion (Watt and Collins, 2008). Nonetheless, there is good evidence that desmosomal cadherins regulate differentiation (Getsios et al., 2009). Dsg1 (desmoglein 1) is first expressed as keratinocytes move upwards out of the basal epidermal layer. Loss of Dsg1 not only impairs intercellular adhesion but also inhibits keratinocyte differentiation in culture. Deletion of the Dsg1 N-terminal domain prevents Dsg1-mediated adhesion but not stimulation of differentiation, indicating that the effects of Dsg1 on differentiation are separable from its effects on intercellular adhesion. Dsg1 promotes differentiation by suppressing ERK MAPK signalling. ERK MAPK inhibition and induction of differentiation markers by DSG1 requires Erbin (ERBB2IP), which binds DSG1 (Harmon et al 2013).

Just as ECM-regulated differentiation on micro-patterned substrates depends on the MAL/SRF transcription factors (Connelly et al., 2010), there is evidence linking MAL to Dsg1-mediated differentiation (Dubash et al 2013). The guanine nucleotide exchange factor (GEF) breakpoint cluster region (Bcr) regulates RhoA activity in keratinocytes. Loss of Bcr reduces differentiation and abrogates MAL/SRF signalling in keratinocytes. Loss of Bcr or MAL reduces Dsg1 expression, and ectopic expression of Dsg1 rescues the effects of due to loss of Bcr or MAL. Taken together, these data identify the GEF Bcr as a regulator of RhoA/MAL signalling in keratinocytes, which in turn promotes differentiation through the desmosomal cadherin Dsg1.

Another desmosome component that plays a role in regulating keratinocyte differentiation is the cytoplasmic protein kazrin, which binds to one of the desmosomal plaque proteins, periplakin (Sevilla et al., 2008). Kazrin overexpression in human epidermal keratinocytes causes profound changes in cell shape and the cytoskeleton and impairs intercellular adhesion by decreasing Rho activity. Kazrin

overexpression also stimulates terminal differentiation, while knockdown of kazrin decreases differentiation and stimulates proliferation without changing total Rho activity. Thus, like Dsg1, kazrin is a dual regulator of intercellular adhesion and differentiation and affects these processes by different mechanisms.

Integration of different signals at cell-cell borders

Just as there is an interplay between ECM adhesion and soluble growth factors in regulating keratinocyte differentiation on micro-patterned islands (Connelly et al., 2010), there is also evidence for integration of growth factor and intercellular adhesion signals at cell-cell borders. EGFR inhibition results in accumulation of intercellular desmoglein 2 (Dsg2) by preventing Dsg2 accumulation in an internalized cytoplasmic pool (Klessner et al., 2009). ADAM10 and ADAM17 regulate shedding and internalization Dsg2, and MMP and EGFR inhibition increase intercellular adhesive strength.

The concept that endocytic trafficking regulates desmosomal function (Brennan et al., 2012) is particularly interesting, in the light of evidence that endocytosis also regulates the function of the Notch ligand Delta-like1 (Dll1) (Watt et al., 2008) and that caveolins are upregulated in epidermal stem cells (Tan et al., 2013a). Human epidermal stem cells express high levels of Dll1, which stimulates neighbouring keratinocytes to differentiate (Watt et al., 2008). Dll1 also promotes integrin dependent ECM adhesion independently of Notch1 signalling (Watt, 2002; Watt et al., 2008).

A number of new techniques have been developed that should greatly facilitate further mechanistic analysis of signalling by proteins that mediate intercellular adhesion. One is to capture keratinocytes on ECM coated micropatterned islands and measure responses to the extracellular domains of adhesive receptors immobilised on polystyrene beads (Figure 3). By using fluorescent beads it is straightforward to compare cells that have no beads, versus one bead or multiple beads within the same population. In this way it should be possible to measure how

keratinocytes integrate stimuli from the ECM and membrane tethered receptors and ligands and to determine whether the response depends on the level of engagement of membrane receptors.

Examples of how such signal integration occur come from studies of mouse sarcoma cells. Cell-fibronectin adhesion decreases the rigidity modulus of the interaction between cells and beads coated with the extracellular domain of E-cadherin. As cell-ECM contact area increases the dynamics of formation of the bead-cell contact decreases, indicating negative feedback from cell-fibronectin to cell-cell adhesive contacts (Al-Kilani et al., 2011). Conversely, cadherin-mediated adhesion stimulates cell spreading on fibronectin-coated hydrogels, increasing cell-fibronectin force by a mechanism that involves signalling via Src and PI3K (Jasaitis et al., 2012).

Even more sophisticated tools for selective and quantitative activation of genetically encoded mechanoreceptors have been developed (Seo et al., 2016) that can be used to examine how spatial segregation and mechanical force cooperate to direct receptor activation dynamics in the case of E-cadherin and Notch. In the case of the WNT pathway, it is possible to control the timing, location and level of signalling by covalently immobilising hydrophobic WNT proteins on a variety of substrates (Lowndes et al., 2016). Together, these types of approach have the potential to reveal how keratinocytes integrate different, and potentially opposing, signals to make cell fate decisions.

Increasing complexity: constructing a micro-epidermis

Capturing single cells on micropatterned islands and hydrogels or modulating the expression and engagement of individual cell surface adhesive receptors has provided information about how the microenvironment influences stem cell fate decisions at unprecedented resolution. It is possible to build on the knowledge gained to study cell-cell interactions in a controlled, quantitative fashion. One approach has been to increase the size of ECM-coated micropatterned islands so that instead of a single cell, small groups of stem cells can be captured. When human

epidermal stem cells are captured on 100 μm diameter circular collagen-coated islands fewer than 10 cells are accommodated, yet within 24h they assemble a stratified micro-epidermis, in which terminally differentiated cells have a central suprabasal location. Just as in the small islands that accommodate single cells (Gautrot et al., 2012), larger islands with a non-adhesive centre still support micro-epidermis assembly. Formation of the tissue requires actin polymerization, adherens junctions and desmosomes, but not myosin II-mediated contractility.

Keratinocytes on 100 μm diameter islands undergo concerted circular motion; however, inhibition of coordinated cell movement does not interfere with epidermis assembly (Gautrot et al., 2012). This contrasts with the observation that human epidermal stem cells in culture undergo collective cell movements that are correlated with their self-renewal ability, the cells with the highest capacity for self-renewal displaying a unique rotational movement that can be identified as early as the two-cell stage colony (Nanba et al., 2015). While the major advantages of the micro-epidermis platform are uniformity and speed of assembly, currently it does not lend itself to analysis of different stem cell subpopulations that differ in expression of specific markers (Tan et al., 2013a) or properties such as cell movement or balanced versus expanding cell growth (Roshan et al., 2016).

Assembling the IFE on a small scale facilitates analysis of the relative importance of cell-ECM and cell-cell adhesion in regulating epidermal assembly. Although the onset of terminal differentiation normally coincides with inhibition of integrin function and expression, thereby ensuring that differentiating cells are selectively expelled from the basal layer, keratinocytes can still initiate terminal differentiation while attached to the culture substrate when junction assembly is inhibited in low calcium medium. Within 6 h of transfer from low calcium to standard medium integrin expression is selectively downregulated in the differentiating cells as they begin to migrate upwards from the basal layer (Hodivala and Watt, 1984). Integrin downregulation can be inhibited by antibody ligation of P- and E-cadherin, indicating crosstalk between the different types of adhesive receptor. At present it does not appear that

ligation of P- and E-cadherin regulates the onset of terminal differentiation, and the role of crosstalk therefore appears to be primarily in coordinating the onset of differentiation with movement upwards from the basal layer.

One of the ways in which the different classes of receptor interact is through the ability of intercellular adhesions to modulate forces transmitted to the ECM (Mertz et al 2013). In the absence of cadherin-mediated adhesion, keratinocytes act independently, and traction forces extend throughout individual colonies. When cadherin-mediated adherens junctions form, traction forces are localised to the colony periphery and cadherin-based adhesions are essential for the observed mechanical co-operativity. In addition, intercellular adhesion controls nuclear position within keratinocytes (Stewart et al., 2015) via a mechanism that involved the linker of nucleoskeleton and cytoskeleton (LINC) complex. The LINC complex contributes to the mechanical integrity of keratinocyte intercellular adhesions and keratinocytes lacking the LINC component Sun2 exhibit aberrant nuclear position in response to adhesion formation, altered desmosome distribution, and mechanically defective adhesions. On micro-patterned surfaces keratinocyte adhesion and spreading regulate the size and shape of the nucleus; nuclear morphology is controlled by keratin filament density around the nucleus, which in turn depends on the cytolinker plectin (Almeida et al., 2015). Thus cross talk between the nucleus, cytoskeleton, and intercellular adhesions is important for epidermal tissue integrity.

By starting with individual keratinocytes and building upwards towards a micro-epidermis it is possible to understand the interactions between cell-cell versus cell-ECM adhesion and how this is integrated via mechanosensing (Yan et al 2015).

Modelling the epidermal-dermal junction

One major difference between mouse and human IFE is that the junction with the underlying dermis is flat in mouse, but undulates in human. The height of the undulations may be physiologically important, since it declines with age (Giangreco

et al., 2010) and increases in hyperproliferative conditions, such as psoriasis (Fraki et al., 1983). Furthermore, in human skin the epidermal stem cell clusters lie in specific locations relative to the undulations (Jones et al., 1995). However, it is not clear from in vivo analysis whether keratinocytes are responding to the topography of the epidermal-junction or to other parameters associated with topography, such as proximity to the skin vasculature.

To explore the role of topography, we developed a series of ECM-coated Polydimethylsiloxane (PDMS) substrates that mimic the topographical features of the human epidermal-dermal interface (Viswanathan et al., 2016). We found that although stem cells can organise into clusters on a flat substrate, when seeded on an undulating surface they are patterned according to topographical features. In addition, by determining the location of cells with highest levels of β1 integrins (the stem cells), cells that were in S phase of the cell cycle, and cells that had initiated terminal differentiation, we found that each of these properties is controlled independently: separate spatial cues determine the locations of stem cells, differentiated cells and proliferating cells. This platform is therefore potentially useful for establishing whether there is patterning of the signalling pathways (such as ERK MAPK and SRF/MAL) that regulate initiation of terminal differentiation in response to ECM-mediated adhesion.

Another approach to recreating the topography of the epidermal-dermal junction is to create collagen-GAG micro-channels (Pins et al., 2000; Bush and Pins, 2012; Clement et al., 2013). On these substrates the stem cells again localise based on topography. However, they are found in the depths of the channels rather than on the tips, mimicking the location of stem cells in the palms and soles of human skin rather than other body sites (Jones et al., 1995). One possibility is that keratinocytes respond to the slope of the channel sides, although it is also possible that bulk stiffness and ECM tethering are factors.

Heterotypic cell-cell interactions

There is no doubt that key components of the epidermal stem cell niche are

contributed by neighbouring cells. This is evident in the case of the epidermal basement membrane, where comparison of cultures of primary human keratinocytes alone or in combination with fibroblasts demonstrates that fibroblasts are the major cellular contributors of the ECM of the dermo-epidermal junction (Benny et al., 2013). Whereas fibroblasts are separated from the epidermis by a basement membrane, keratinocytes and melanocytes make extensive cell-cell contacts. Golan et al (2015) have examined the changes in microenvironment that occur when malignant melanocytes - melanoma cells – switch from radial to vertical growth within the epidermis. They found that direct contact of melanoma cells with the suprabasal keratinocytes triggers vertical invasion via activation of Notch signaling.

Controlling the interactions between different cell types at high resolution can be achieved using a recently described platform in which DNA-programmed adhesion can specify the number and initial position of up to four distinct cell types and control over cell-contact time (Chen et al., 2016). In the case of neural stem cells this has revealed a signalling hierarchy involving Dll1 and ephrin-B2 ligands, whereby the stem cell compartment is maintained by the simultaneous presentation of both signals.

Considerable progress has also been made in modulating fibroblast behaviour by developing new materials that mimic the fibrillar ECM of the dermis (Baker et al., 2015) and the differences in the dermal ECM in healthy and wounded skin (Sakar et al., 2016) and following scar formation (Dingal et al., 2015). These studies are becoming more sophisticated by including co-cultures of fibroblasts and keratinocytes (Planz et al., 2016; Wang et al., 2016) or more complex combinations of keratinocytes, fibroblasts and endothelial cells (An et al., 2015). Together these studies can be used to examine how different cell types collaborate during wound healing. In future, it will be interesting to populate the dermal ECM with different fibroblast subpopulations, since studies in mouse skin have shown that fibroblasts represent distinct cell lineages with different functions that communicate

reciprocally with the epidermis via different signalling molecules (Lichtenberger et al., 2016).

Conclusions

Our understanding of how the interplay between different intrinsic and extrinsic signals regulates epidermal cell behaviour has progressed considerably through the use of micro-patterned substrates. In parallel, the clinical applications of stem cells and regenerative medicine are an area of intense investigation, and therapies targeting the stem cell niche are becoming both feasible and appealing (Lane et al., 2014). The possible approaches to understanding the epidermal stem cell niche are expanding rapidly. In particular, high throughput screens of nano-scale topographical features (Unadkat et al., 2011; Reimer et al., 2016) and combinations of ECM proteins and tethered soluble factors (Gobaa et al., 2011; Desai et al 2014) lend themselves to studies of keratinocyte cell fate decisions at the single cell level. The success of these types of approaches lies on high content imaging platforms and a robust computational framework for analysing the results.

Most of the assays described in this review are endpoint assays. However, micro-patterned surfaces lend themselves to live cell recording (Gautrot et al., 2012; Kerz et al., 2016) and the use of fluorescent sensors of key signalling pathways and proteins (Gautrot et al., 2014). In vivo imaging of Erk MAPK activity in the epidermis of living mice has already shown that signalling is not cell autonomous but coordinated over many cell diameters (Hiratsuka et al., 2015) and combining key reporters with markers of different stem cell states (Tan et al., 2013) is an exciting prospect. In addition to understanding the dynamics of signalling, it is possible to create dynamic substrates, such as hydrogels with tethered EGF that can be exposed or released (Cambria et al., 2015). There is also tremendous potential to apply micro-patterned islands and other tools to gain a deeper understanding of complex and monogenic diseases, thereby revealing defects that can potentially be targeted therapeutically, as in the case of squamous cell carcinoma (Gautrot et al., 2012).

Finally, it is important to point out that one of the reasons why the epidermal stem cell field has blossomed in the last 25 years is through the use of mouse models in which epidermal-specific promoters can be used to target gene expression to particular cell layers and gene knockout technology can be used to build models of human disease (Fuchs and Coulombe, 1992). At first it appeared difficult to reconcile the in vitro findings obtained using human keratinocytes with the in vivo studies of mouse epidermis. One issue was that the complexity of the multiple differentiation lineages in mouse epidermis could not be adequately modeled in cultured human epidermis. Another was that the complex phenotypes attributed to individual epidermal genes in vivo were often due to secondary effects on the immune system, such as inflammation-mediated epidermal hyperproliferation. We now, however, have a more holistic approach to epidermal research that will only grow as tools for manipulating human epidermis in mouse models and generating multiple skin cell types from pluripotent stem cells become even more sophisticated (Higgins et al., 2013; Gledhill et al., 2015; Takagi et al., 2016).

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Figure legends

Figure 1. Mimicking extrinsic signals that regulate epidermal stem cell fate in vitro. Micrograph shows section of adult mouse back skin in which Pdgfra-positive fibroblasts are labelled with nuclear GFP (green fluorescence), α8-integrin positive cells of the arrector pili muscle are labelled in red, and nuclei are counter-stained with DAPI (blue).

Figure 2. Scanning electron micrograph of a human keratinocyte adhering to a 20 μm diameter micro-patterned island. Scale bar: 10 μm.

Figure 3. Modulating intercellular adhesion on micro-patterned substrates with attached E-cadherin coated beads. Human keratinocytes seeded on 50 μm diameter micro-patterned islands are incubated with E-cadherin-FC beads. Arrows in phase contrast panel show cells with attached beads. Middle and right hand panels show one field of cells, indicating islands with more than one adherent cell and cell with attached beads.

Figure 1

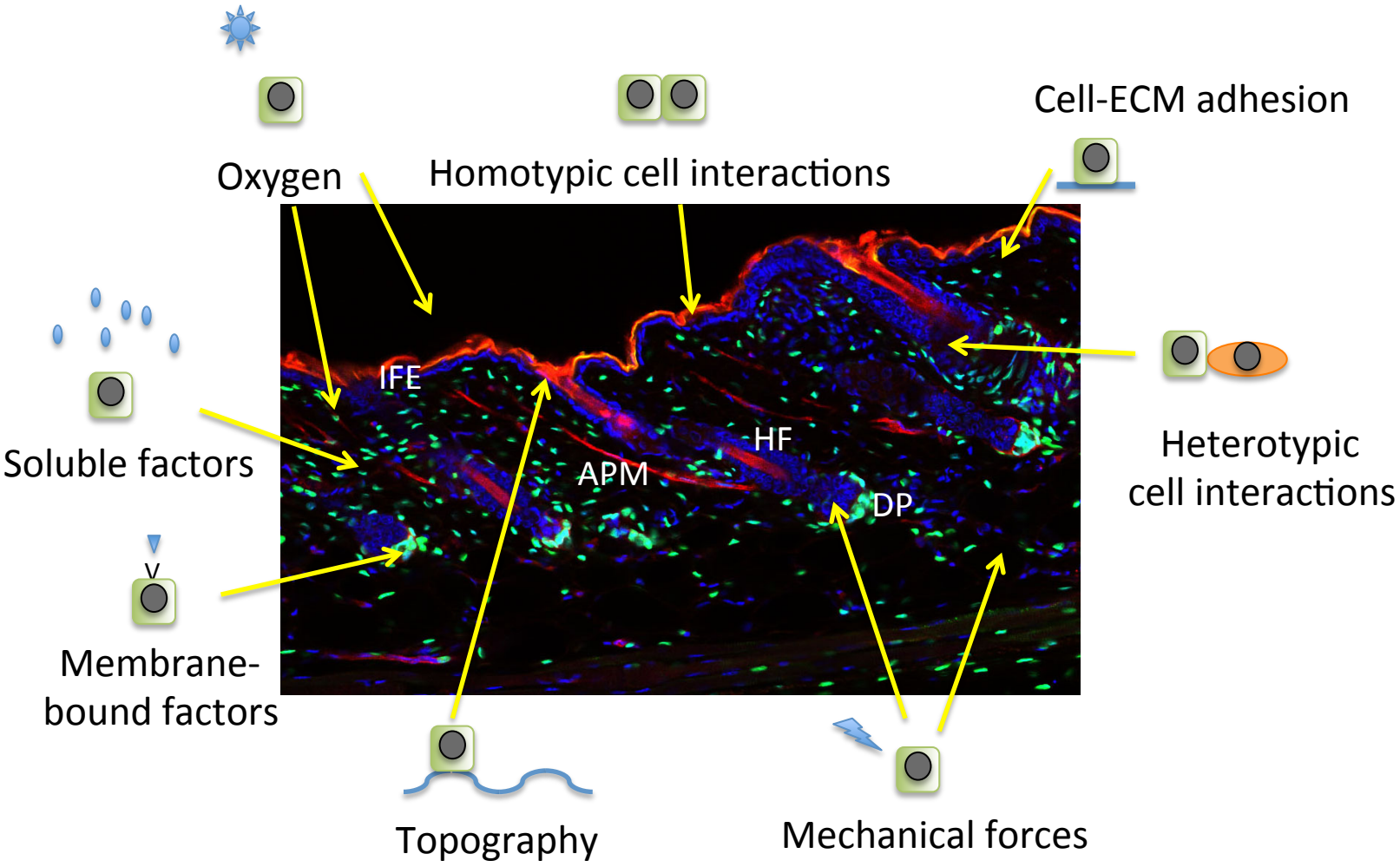
Oxygen
Homotypic cell interactions
Cell-ECM adhesion
IFE
HF
APM
DP
Soluble factors
Heterotypic
cell interactions
Membrane-
bound factors
Topography
Mechanical forces

Figure 2

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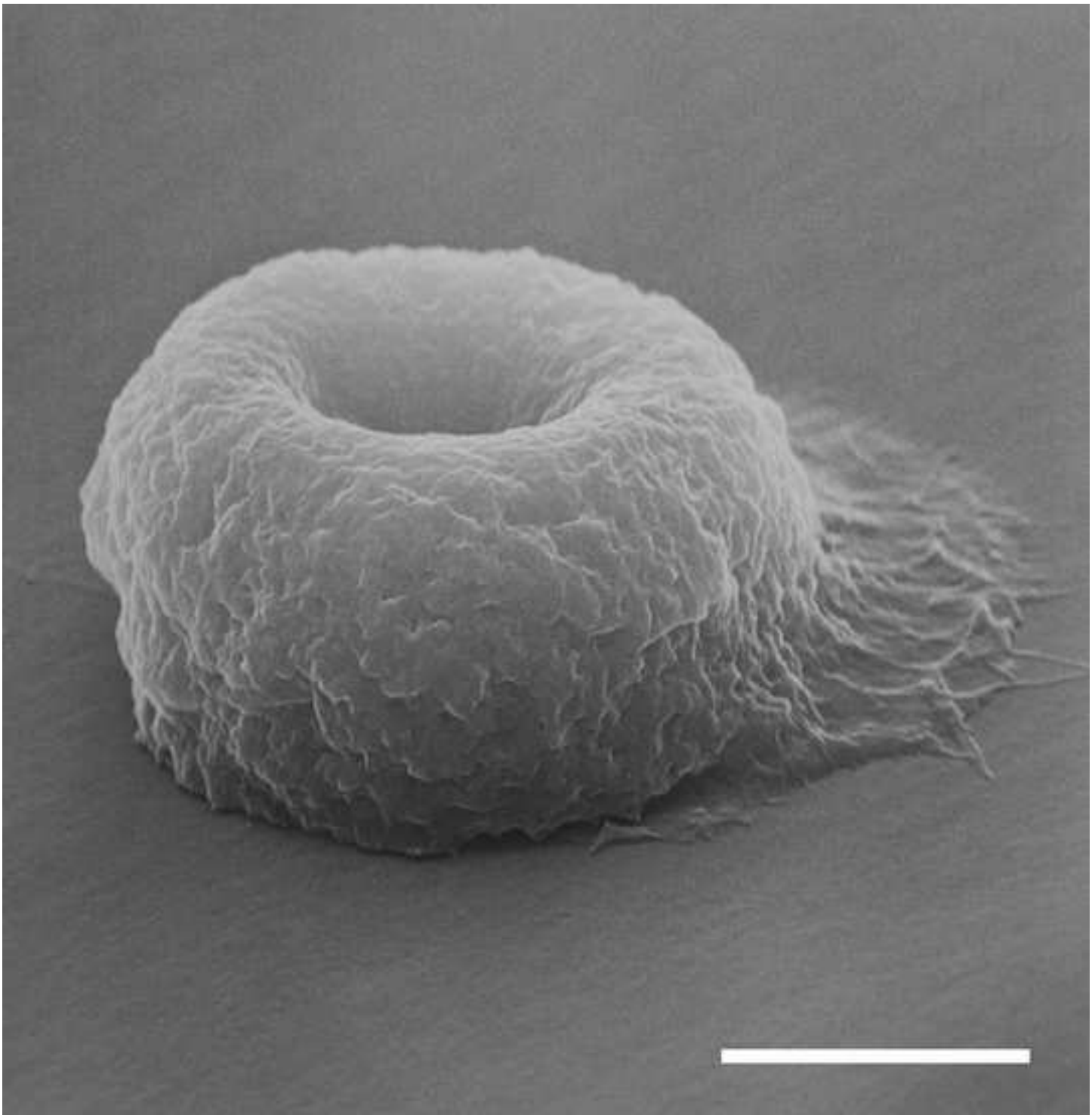

Figure 3